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(54) Quantitative Analysis Apparatus and Method

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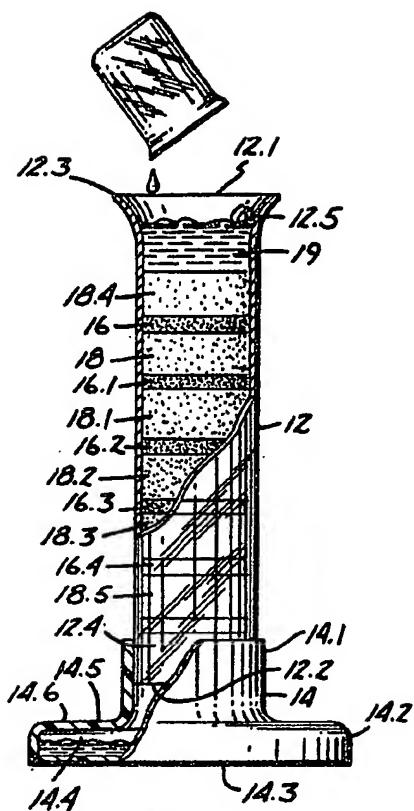


Fig. 1

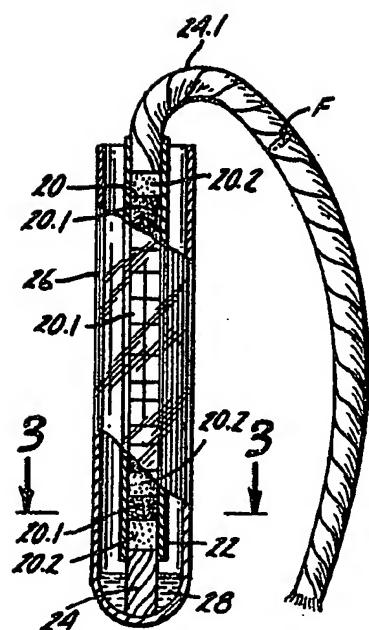


Fig. 2

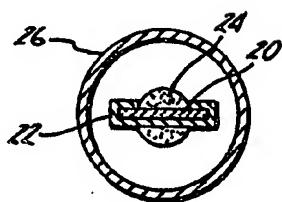


Fig. 3

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Fig.5

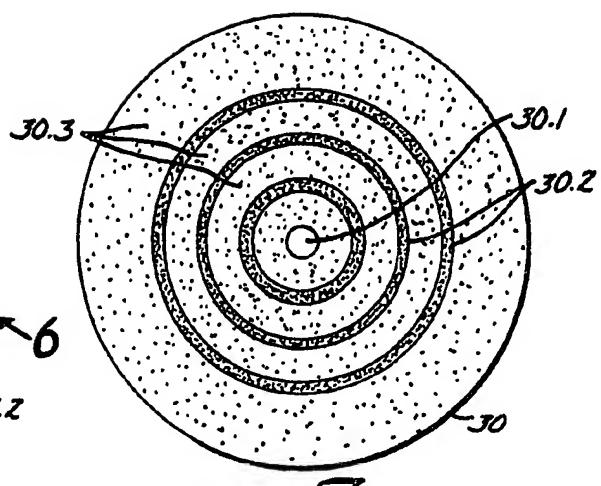
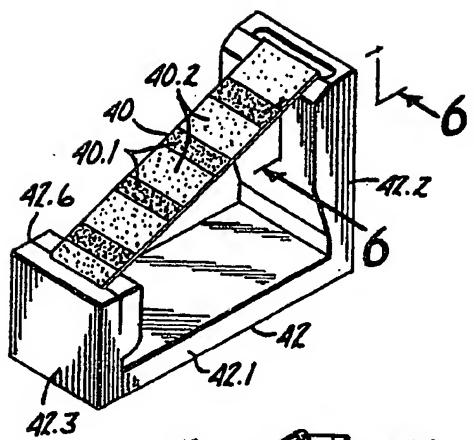


Fig.4

Fig.6

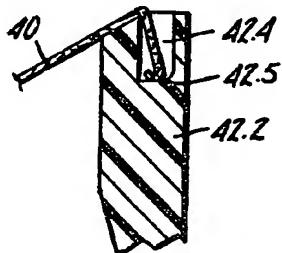
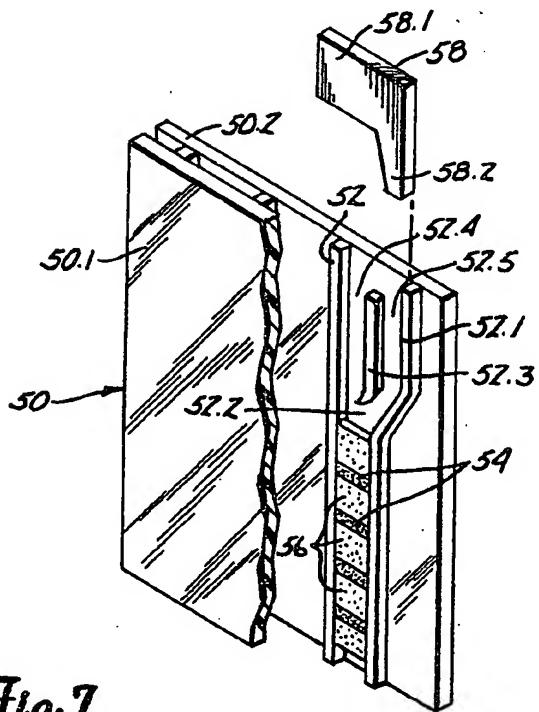


Fig.7



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ABSTRACT

A method and apparatus for the quantitative determination of an analyte in a liquid employs a liquid-permeable solid medium defining a liquid flow path. The medium includes a number of reactant-containing reaction zones spaced apart along the flow path and in which reaction occurs with the analyte or an analyte derivative (e.g., a labeled analyte) to result in the formation of a predetermined product. Detector means are employed to detect analyte, analyte derivative, reactant or predetermined product in the reaction zones, the number of such zones in which such detection occurs indicating the amount of analyte in the liquid.

QUANTITATIVE ANALYSIS APPARATUS AND METHOD

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FIELD OF THE INVENTION

The invention is in the field of quantitative chemical analysis, and is particularly useful in the detection and analysis of small amounts of chemical substance in such biological fluids as milk, blood, urine, etc.

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DESCRIPTION OF THE PRIOR ART

Procedures for quantitatively determining the concentration of chemical substances in solutions are legion. Many of these procedures are long and tedious, and are highly susceptible to human error. Many procedures involve the reaction of the chemical moiety - the analyte - to be detected with a reactant to form a product, the procedures including a step of determining the amount of reactant that is consumed (e.g., as in titrations), or the amount of product that is produced (e.g., as by measuring the absorption of light by the product of a chromogenic reaction), or as by measuring the amount of the chemical moiety or reaction product that can be separated from the solution (e.g., by distillation), etc. Some quantitative analysis procedures, such as are used in radioimmunoassays, involve competitive reactions between labeled analytes (e.g., labeled with radioisotopes of iodine, enzymes, or fluorescent, chromogenic or fluorogenic molecules) in known quantities and unknown amounts of

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1 unlabeled analytes, the amount of analyte in the un-
known solution being related to the measured radioac-
tivity or other property of a specimen resulting from
the test after suitably separating the reacted or
bound analyte from the unreacted or unbound analyte,
or through properties of the bound and unbound labeled
analyte that permit them to be distinguished. Many of
such procedures involve changes in color (as when
chemical indicators are employed that respond by color
10 changes to differences in hydrogen ion concentration),
or in turbidity (as when the procedure involves the
formation of a solid reaction product).

20 Certain analyses involve the passage of a
fluid, such as air, through a column containing a re-
actant which may change color upon contact with an
ingredient of the air. For example, U.S. Patent
3,286,506 describes a gas analyzing technique in which
a measured amount of gas is passed through a glass
cartridge containing an indicator, the amount of gas
to be detected being proportional to the amount of
indicator within the column that changes color. Sim-
ilar devices are shown in U.S. Patents 3,312,527 and
3,545,930.

30 There is a recurring trend in the field to pro-
vide analytical procedures which are characterized by
speed, simplicity, and by a reduction in the vulnar-
ability of such procedures to human error. Simple,
rapid tests, for example, have been marketed for de-
termining the approximate level of blood sugar for
diabetics. Such tests, however, often are relatively
imprecise. It would be highly desirable to provide a
quantitative test for chemical moieties that on the
one hand would be characterized by high sensitivity
and that yet on the other hand would be characterized
by simplicity, rapidity and relative freedom from
human error.

1 SUMMARY OF THE INVENTION

In one embodiment, the invention provides an apparatus for the quantitative analysis of a chemically reactive substance (hereafter referred to as an "analyte"), in a carrier fluid such as a liquid. The apparatus includes a fluid-permeable solid medium that has a predetermined number of successive, spaced reaction zones and which defines a path for fluid flow sequentially through such zones. "Fluid" herein is typified as a liquid. Predetermined quantities of a reactant are bound to the solid medium in such zones and are capable of reaction with the analyte or with an analyte derivative, to result in the formation of a predetermined product. The apparatus may further include detector means for detecting, in the spaced zones, the presence of the analyte or its derivative, the reactant, or the predetermined product resulting from the reaction between the analyte or its derivative and the reactant. In addition, the apparatus may include means for suppressing the detectability of trace amounts of the analyte or its derivative, the reactant, or the predetermined product resulting from the reaction between the analyte or its derivative and the reactant.

As used herein, the terms "reactant", "reactive" and the like when used in connection with the reaction between the analyte or its derivative and the reactant refers to the ability of the reactant to react, by covalent or hydrogen bonding or by any other means, with the analyte or its derivative to form or result in the formation of a predetermined product. That is, such terms are used in their broadest sense as referring to the ability of the reactant to in any way act upon, be acted upon, or interact with the analyte or analyte derivative in a manner that detectably alters the analyte or its derivative, the reactant or

1 both to thereby result in the formation of a reaction
product. Similarly, "reaction product" means any
product resulting from the reaction of the analyte or
its derivative and the reactant and that is detectably
different from both. "Analyte derivative" means a
chemical moiety derived from an analyte, and desirably
is a tagged or labeled form of the analyte as may be
employed in analytical procedures involving competing
reactions between an analyte and its tagged or labeled
derivative.

10 In the apparatus of the invention, the reactant
is bound to the permeable solid medium in the successive,
spaced zones through which the analyte passes.
A procedure employing the apparatus may take the form
in which the analyte or its derivative, as it passes
through the reaction zones, becomes bound to the re-
actant and the presence of the analyte or its deriva-
tive within the reaction zones is detected, as by
color change or the like. Similarly, in a slightly
20 modified embodiment, the analyte or its derivative may
react with the reactant to result in the formation of
a product that itself remains bound in the reaction
zones, and the product itself is then detected. In
these embodiments, one may determine with considerable
precision the concentration of the analyte by detect-
ing how many of the successive reaction zones, begin-
ning with the upstream zone, show the presence of the
analyte or its derivative, or of the product resulting
from the reaction between the reactant and the analyte
30 or analyte derivative. In another embodiment, the
reactant that is bound to the permeable solid medium
may itself be capable of detection by suitable detec-
tion means and may be disabled from such detection
when reacted with an analyte or analyte derivative.
In this manner, as the analyte or analyte-analyte

1 derivative composition passes through successive reaction zones, the reactant in the successive zones is disabled from such detection until substantially all of the analyte or analyte-analyte derivative composition has been exhausted, while remaining downstream reaction zones still contain reactant that can be detected. In a modified form, the reaction between the analyte or analyte derivative and the reactant may cause the latter to become unbound from the solid
10 medium to which it was attached and hence be washed from the successive zones. When the analyte or analyte derivative or both has thus been exhausted, subsequent or downstream reaction will display reactant that is yet bound to the permeable medium and which can be detected. In such embodiments, one may count the number of zones in which the reactant has been disabled beginning with the upstream zone.

As used herein, "analyte" refers not only to the particular chemical moiety for which analysis is desired, but also to chemical moieties that are reaction products of the moiety to be determined with another chemical moiety. For example, a biological fluid containing an unknown amount of a chemical moiety may be reacted in solution or otherwise with another chemical moiety to provide a product, the concentration of which is related to the initial concentration of the chemical moiety to be measured. The resulting product, then, may become the "analyte" for use in the apparatus and method of the invention.
20 Accordingly, "analyte" refers to any chemical moiety which is to be measured quantitatively.

In a preferred embodiment, the invention employs immunochemical reactions in which the analyte and the reactant represent different parts of a specific ligand-antibody (antiligand) binding pair.

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DESCRIPTION OF THE DRAWINGS

Figure 1 is a broken-away view, in partial cross-section, showing an apparatus of the invention;

Figure 2 is a broken-away view, in partial cross-section, showing another apparatus of the invention;

Figure 3 is a cross-sectional view taken along line 3-3 of Figure 2;

10

Figure 4 is a plan view of another embodiment of an apparatus of the invention;

Figure 5 is a perspective view of yet another test apparatus of the invention;

Figure 6 is a broken-away cross sectional view taken along line 6-6 of Figure 5; and

Figure 7 is a perspective, broken-away view of another test apparatus of the invention.

DETAILED DESCRIPTION

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With reference to Figure 1, a transparent, hollow column of glass or the like is designated 12 and has open top and bottom ends (12.1, 12.2). The top end (12.1) preferably is flared outwardly as shown at (12.3). A standard (14) is provided at the bottom end of the column, and may have a hollow, upstanding portion (14.1) into which the bottom portion (12.4) of the column may be snugly fitted as by a press fit.

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B The standard includes a relatively wide bottom portion (14.2) having a flat, horizontal surface such as a table. The interior (14.4) of the standard preferably is hollow, and an upper wall (14.5) of the standard preferably is provided with a breathing aperture (14.6) so as to permit air to escape from the column when liquid is poured into the upper end (12.1) of the column. The aperture (14.6) may, if desired, be fitted with a loose, porous plug, such as a cotton plug, to retard leakage from the device when it has been disposed of in a trash container or the like. It

1 may also, if desired, be fitted with a flexible tubing
which may connect it with a pump (e.g., peristaltic,
syringe drive withdrawal, etc.) typifying flow control
means to control the flow rate of the liquid through
the assay column.

B Within the column are positioned successive,
spaced reaction zones (16, 16.1, 16.2, 16.3, ^{16.4}, etc.),
occupied by a permeable solid medium such as beaded
agarose, beaded polyacrylamide, porous glass, cellu-
lose or other materials permeable to liquid and com-
patible with the analyte, analyte derivative, reactant
and detector means. To the medium in the reaction
zones is bound a reactant, as will be described more
fully below. The interior of the column, as will now
be understood, describes a generally vertical liquid
flow path, and the permeable solid medium positioned
in the reaction zones desirably occupies the entire
cross-section of the flow path. Between the spaced
reaction zones are positioned preferably nonreactive
20 spacer layers (18, 18.1, 18.2, 18.3 and so on) of a
liquid-permeable solid medium through which liquid may
flow, the spacer layers preferably being in intimate
contact with the reaction zones. The spacer layers
desirably are of the same permeable solid medium as
the reaction zones, and, preferably, spacer layers
18.4, 18.5 are provided at the top and bottom of the
columns as well so that each reaction zone is sand-
wiched between spacer layers. At its upper end, the
column (12) may be provided with an aperture (12.5)
30 spaced a given distance above the spacer layer (18.4)
so as to provide a predetermined volume between the
aperture and top surface of the spacer layer. In a
known manner, as a liquid (represented as (19) in the
drawing) is poured into the open upper end (12.1) of
the column, it will occupy the open volume at the top

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1 **B** of the column and any amount of the liquid ~~(20)~~ in excess of that desired may escape outwardly through the aperture (12.5), thereby insuring that no more than a given, predetermined amount of the liquid passes downwardly in the column. The space ¹⁹ ~~(20)~~ may, if desired, be filled or partially filled with a porous, nonreactive material such as glass wool or similar material to avoid splashing of the liquid within the upper end of the column.

10 To the liquid-permeable solid medium within the spaced reaction zones (16, 16.1, etc.) is bound a reactant that is reactive with a given analyte or analyte derivative to form a product, all in accordance with the above definitions and as exemplified herein. As a typical example, the reactant and analyte may be so chosen that the analyte or its derivative becomes chemically attached to the reactant as the solution ~~(20)~~ of analyte or analyte and analyte derivative solution (the "test solution") passes downwardly through the column, care being taken that the total amount of reactant within the column is in excess of that required to so react with the expected quantity of analyte and analyte derivative in the solution. After the test solution has begun its passage downwardly through the column, a wash solution, typically distilled and deionized water, optionally may be poured into the open end of the column to further aid the downward passage of the test solution through the column. Finally, an indicator or detector material that detects the presence of analyte or analyte derivative, reaction product or reactant, as by causing a color change within the zones (16, 16.1 and so on) may be poured into the upper end of the column. As the test solution flows downwardly through the column, predetermined amounts of the analyte or its derivative are reacted with or bound to the reactant in each such

20

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1 layer until the analyte or its derivative have been
exhausted from the solution. The concentration of
analyte in the solution can be determined by merely
counting the number of successive zones, beginning at
B the top of the column, that have changed color. In
another embodiment, the reactant that is bound to the
medium in the zones (16, 16.1 and so on) may be de-
activated or disabled by reaction with the analyte
or analyte derivative or both, and the detector which
10 is employed may determine, as by a color change, the
presence of nondisabled reactant. In this embodiment,
the reactant in successive zones contacted by the test
solution will be disabled until analyte and analyte
derivative have been exhausted from the solution.
Upon detecting which of the reaction zones contain
reactant that has not been disabled, one may determine
the concentration of analyte in the solution by count-
ing the number of zones beginning at the top of the
column in which reactant is not detected. Of course,
20 in this embodiment as in the embodiment set out above,
one may also count the number of zones beginning with
the bottom of the column as well.

Another physical embodiment of the apparatus
of the invention is shown in Figures 2 and 3 in which
"wicking", or upward capillary flow of a liquid
through a strip of filter paper or similar material is
employed, the strip having successive, spaced reaction
zones. In this embodiment, the permeable solid medium
may take the form of a strip of filter paper, which is
30 designated generally as (20) in Figures 2 and 3.
Within spaced zones (20.1) of the filter paper strip
is bound a reactant, as above-described, the spaced
zones being separated by spacer layers or sections
(20.2). One method of preparing the strip (20) in-
volves binding a reactant to small, individual rec-
tangular paper filter pieces, and then alternating

1 these pieces, which form the reaction zones, with
similar pieces of filter paper that do not contain the
reactant, the alternating pieces of filter paper being
held together, for example, by a thin strip of ad-
hesive tape. Other, more sophisticated methods of
manufacture will be apparent to those skilled in the
art.

10 As shown in Figures 2 and 3, the strip (20) of
filter paper may be positioned in an elongated plastic
holder (22) having a generally C-shaped cross-section.
The bottom of the holder is adapted to receive the end
of a wick (24) which may consist of twisted strands of
cotton or other fibrous material. A similar wick
(24.1) is received in the upper end of the plastic
holder. The ends of the wicks (24, 24.1) contact the
ends of the filter strip (20). As shown in Figure 2,
the upper and lower sections of the filter paper strip
which come into contact with the wicks (24, 24.1), are
spacer layers (20.2) so that each reaction zone (20.1)
is sandwiched between spacer layers (20.2). The fil-
ter paper strip and holder are adapted for insertion
into a test tube (26) or other container so that the
lower wick (24) contacts the bottom of the test tube
and the upper wick (24.1) extends out of the test tube
and then downwardly toward its bottom, all as shown in
Figure 2. A test solution (28) placed in the bottom
of the test tube (26) is thus caused to flow by capil-
lary action upwardly through the length of the filter
paper strip, successively contacting the reaction
20 zones (20.1) in a manner analogous to the flow of test
solution through the column depicted in Figure 1. As
will be described more fully below, the filter paper
strip and holder can be moved from one test tube to
another so that different solutions can be caused to
sequentially flow through its length.

1 Referring now to Figure 4, an apparatus of the
invention is embodied in a disc of permeable, solid
medium such as filter paper, porous glass, etc. (30).
The disc (30) may be placed horizontally in a suitable
container such as a petri dish. At its center, the
disc (30) is provided with a well (30.1) to receive a
test solution or other solution. Reaction zones,
spaced radially from the well (30.1), are shown as an-
nular rings (30.2), and are separated from one another
by spacer layers also in the form of annular rings
10 (30.3). Spacer layers preferably form the innermost
and outermost rings of the disc. The reaction zones
(30.2) and the spacer layers (30.3) are concentric.
Test solution that is admitted to the central well
(30.1) is thus carried radially outwardly of the well
by capillary action or by diffusion, aided by centrif-
ugal force if desired, the test solution successively
passing through the spaced reaction zones (30.2).

20 Figures 5 and 6 show another embodiment of a
device of the invention. The device includes a filter
paper strip (40) similar to that of Figures 2 and 3
and containing spaced reaction zones (40.1) separated
by spacer layers (40.2). A holder, preferably of
plastic, is designated (42) and has a flat base (42.1)
with upwardly extending legs (42.2, 42.3) carried at
its ends. The leg (42.2) is provided with an upwardly
open well (42.4) into which may be inserted the upper
end of the filter paper strip (40), care being taken
that the end (42.5) of the filter paper extends down-
30 wardly to the floor of the well. The strip of filter
paper extends obliquely downwardly from the well, and
its lower end is captured in a slot (42.6) formed in
the leg (42.3). In use, the test solution or other
solution is placed in the well (42.4), and is carried
downwardly of the strip by both gravity and capillary

1 action, the solution sequentially encountering the
spaced reaction zones (40.1).

Figure 7 shows yet another embodiment of a device of the invention which can be used for multiple concurrent tests. The device, designated (50), includes a pair of spaced plates (50.1, 50.2). Referring to the right-hand portion of Figure 7, the space between the plates is divided into generally vertical channels by means of elongated spacers (52, 52.1). As shown in the drawing, the channel (52.2) formed by the spacers has a wide upper section and a narrow lower section. The lower section is provided with a series of vertically spaced reaction zones (54) comprising a liquid-permeable solid medium to which is bound a reactant, the medium being any of those described above. Between the reaction zones, are placed spacer layers (56), the spacer layers sandwiching between them the reaction zones (54). Between the spacers (52, 52.1), at the upper end of the channel is placed an elongated vertical divider (52.3) which divides the upper portion of the channel into two sections (52.4) and (52.5). A plug (58), which may be made of the same material as the spacers, has an upper, finger-gripping portion (58.1) and a lower, tapering plug portion (58.2) adapted to be inserted in the channel (52.5). The flat surfaces of each of the spacers and plug, of course, contact the facing surfaces of both glass plates to prevent leakage of material from the channels.

In use, a solution such as a test solution is poured into the upper end of the channels formed by the spacers (52, 52.1), and the plug (58) is then inserted to provide an air-tight upper seal in the one channel (52.5). As a result, liquid in the other channel (52.4) preferentially flows downwardly through the reaction zones and spacer layers. When the liquid

1 level in the channel (52.4) falls below the lower end
of the spacer (52.3), air can bubble upwardly through
the channel (52.5), permitting the contents of that
channel to empty downwardly through the reaction zones
as well. In this manner, the sequential flow of liq-
uid, first from channel (52.4) and then from channel
(52.5), is rendered automatic. Preferably, one of the
plates (e.g., plate 50.1) is transparent so that the
results of any color change in the reaction zones may
readily be observed. The other plate (50.2) may be
transparent or may be of an opaque white or other
light color to serve as a background against which
color changes can readily be seen.

ANALYTES-REACTANTS

Analytes that can be detected in accordance
with the present invention include substantially all
chemical substances that are reactive with a reactant
to form a product, as above discussed. It will be
understood that the invention is not limited to any
particular analyte or reactant, but is useful for sub-
stantially any analyte-reactant combination.

Many analytes may be analyzed simply by adapt-
ing known chemical reactions to the invention.

For example, carbon dioxide may be analyzed
with phenolphthalein at a slightly alkaline pH. Cal-
cium ion may be analyzed utilizing a reactant compris-
ing calmodulin and mammalian phosphodiesterase or
another calmodulin-sensitive enzyme (Maekawa and Abe,
Biochemical and Biophysical Research Communications
30 97:621 (1980)). Ferrous ion may be analyzed utiliz-
ing, as a reactant, a ferrocene derivative (Katz, et
al, J. Am. Chem. Soc. 104:346 (1982)). A large number
of additional examples may be selected from the list
of organic analytical reagents compiled by John H. Yoe
in Handbook of Chemistry and Physics, p.D 126-129,

1 57th Edition, Robert C. Weast, Ed., CRC Press, Cleve-
land, 1976, and in other references cited therein.

Typical analyte-reactant pairs selected from
the field of organic chemistry similarly may be chosen
by adapting known chemical reactions to the inven-
tion. For example, almost any phenol may be analyzed
with Gibbs Reagent (2,6-dichloro-p-benzoquinone-4-
chlorimine) (Dacre, J., Analytical Chemistry 43:589
(1971)). A reagent for Indoles is p-dimethylamino-
benzaldehyde (Fieser and Fieser, Reagents For Organic
Synthesis, Volume 1, p.273, John Wiley & Sons, Inc.,
New York, (1967)). The last-mentioned reference also
shows the use of phenylhydrazine as a reactant for
cortisone and similar steroids, and the use of sulfo-
acetic acid as a reactant in the Liebermann - Burchard
test for unsaturated sterols. Amino acids and ammoni-
um salts may be analyzed using the reagent ninhydrin.
(indane-1,2,3-trione hydrate) (Pasto, et al Organic
Structure Determination, p.429, Prentice-Hall, Inc.,
Englewood Cliffs, New Jersey, 1969). Reducing sugar
may be measured with Red Tetrazolium (2,3,5-triphenyl
-2H-tetrazolium Chloride) (Fieser, Organic Experi-
ments, p.135, Raytheon Education Co., Lexington,
Massachusetts, 1968).

Various other analyte reagent pairs may be se-
lected from the field of chemistry for adaptation to
the invention from such reference works as: Schuurs,
et al, U.S. Patent 3,654,090 (Enzyme-Linked Immuno-
sorbent Assay); Kay, U.S. Patent 3,789,116 (Fluores-
cent Labeled Antibody Reagents); Rubenstein, et al,
U.S. Patent 3,817,837 (Homogeneous Enzyme Immuno-
assay); Ling, U.S. Patent 3,867,517 (Radioimmuno-
assay); Giaever, U.S. Patent 3,906,490 (Radial Immuno-
diffusion); Ullman, U.S. Patent 3,996,345 (Floures-
cence Quenching Homogenous Immunoassay); Maggio, U.S.
Patent 4,233,402 (Enzyme Channeling Homogeneous Enzyme

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Immunoassay); Boguslaski, et al, Canadian Patent 1,082,577 (Hapten-Cofactor Homogeneous Enzyme Immunoassay); Schonfeld, H., Ed., "New Developments in Immunoassays", Antibiotics and Chemotherapy, Volume 26, 1979; O'Sullivan, et al, "Enzyme Immunoassays: A Review", Annals of Clinical Biochemistry 16:221 (1979); Schuurs, et al, Enzyme Immunoassay, Clin. Chim. Acta. 81:1 (1977); Feldmann, et al, Eds., First International Symposium On Immunoenzymatic Techniques, INSERM Symp.

10 No. 2, North Holland Publishing Co., Amsterdam, 1976; Williams, et al, Methods in Immunology and Immunochemistry, Volume 3, Academic Press, New York, 1971; and Yalow, et al, J. Clin. Invest. 39:1157 (1960).

Yet other analyte-reactant pairs may be found in: reference works such as Feigl, F., Spot Tests in Inorganic Analysis, 6th edition, Elsevier Publishing Co., New York, 1972; Feigl, Fritz, Spot Tests in Organic Analysis, 7th edition, Elsevier Publishing Co., New York, 1966; Snell, F. and Snell, C., Colorimetric Methods of Analysis, Vols. 1-4AAA, Van Nostrand Reinhold Co., New York, 1967-74; and Braibanti, A., Ed. Bioenergetics and Thermodynamics: Model Systems - Synthetic and Natural Chelates and Macrocycles as Models for Biological and Pharmaceutical Studies, D. Reidel Publishing Co., Boston, 1980.

20 Of particular importance to the instant invention are those analyte-reactant combinations that form specific binding pairs of which one is an antibody and in which the other is a ligand to which the antibody is specific.

30 Such immunochemical reactant pairs are well-known in the art, and a wide variety of tests have been devised to detect the presence of quantity or



1 both of an analyte, particularly when the analyte is
present only in exceedingly small concentrations.
Reference is made to the above-identified patents and
publications.

DETECTORS

10 The detectors useful in the invention are capable of detecting the presence in the successive reaction zones, of analyte, analyte derivatives, reactants, or the predetermined reaction product, all as described above. The means of detection may take various forms. In the preferred embodiment, detection is signaled by a change of color, or a lack of a change of color, in the respective reaction zones of the apparatus. However, detection may be signaled by other means as well, such as by luminescence or fluorescence of the zones, radioactivity of the zones, etc. For many reactions, detection is signaled by a change in pH, and the detector may hence take the form of a pH color indicator such as phenolphthalein, Nile Blue A, Thymol Blue, and Methyl Violet. In other tests, one may detect the presence or absence of the appropriate chemical moiety in a reaction zone by observing whether a solid reaction product has settled upon the succeeding spacer layer. Various detector mechanisms are known to the art, and need not be described in detail. In the preferred embodiment, however, which makes use of immunochemical reaction between the analyte or the analyte and its derivative and the reactant, often very small concentrations of analyte are to be measured and accordingly a magnifying or amplifying mechanism may suitably be employed. One such mechanism makes use of enzymes to promote the reaction of a reaction product with a detector moiety to in turn provide a visual color indication. For example, the analyte to be tested may be provided in admixture with a known quantity of an analyte-glucose

1 oxidase conjugate as an analyte derivative, the
spaced, sequential reaction zones of the apparatus
containing an antibody specific to the analyte. A
signal generating system, such as horseradish peroxy-
dase bound to the antibody in the permeable solid
medium in such zones and a chromogenic material such
as o-dianisidine (added, with glucose, to the test
solution) can be employed. The addition of the test
solution, containing the analyte, the analyte-glucose
10 oxidase conjugate, glucose, catalase and o-dianisidine,
is then flowed through the apparatus, such as the
column depicted in Figure 1. The analyte and the ana-
lyte-glucose oxidase conjugate compete for binding
sites on the bound antibody, thereby resulting in a
color formation due to the reaction of the o-dianisi-
dine with hydrogen peroxide produced by the glucose
oxidase - catalyzed reaction of oxygen with glucose.
Unreacted analyte and analyte-glucose oxidase conju-
gate flow to successive zones until the admixture has
20 been exhausted of analyte and analyte-glucose oxidase
conjugate. A variety of modifications of this proce-
dure, of course, are known to the art.

Example I

The chromogen 5,5' [3-(2-Pyridyl)-1,2,4-
triazine-5,6-diyl]bis-2-furansulfonic acid, disodium
salt, ("Ferene", a trademarked product of Chemical
Dynamics Corp.) is used for the determination of serum
iron in soluble assays through measurement of the
absorbance at 593nm, at which wavelength any inter-
ference from other pigments in the serum should be
30 minimized. It may be covalently coupled to useful
carrier derivatives through nitration, reduction,
diazotization and diazonium coupling to proteins (such
as albumin) immobilized on agarose beads, paper strips
or other suitable permeable solid medium. This im-
mobilized signal-generating reagent (chromogenic

1 chelating agent) is physically arranged in sequential spaced layers or bands (reaction zones) through which the test fluid will migrate.

Small columns are prepared from silanized Pasteur pipettes by breaking off both ends, attaching a short piece of tubing to the bottom (constricted) end and inserting glass wool plugs in the tube bottoms. The columns are packed by sequential insertion of layers of agarose-Ferene separated by layers of 10 unmodified agarose. Typically, 0.4 ml of a 1:1 agarose suspension is applied directly above the support, followed by alternating layers of 50 microliters of a 1:1 suspension of agarose-Ferene and 0.2 ml of agarose suspension. After each addition to the column, the walls of the columns are rinsed with phosphate-buffered saline (PBS) and the solution above the gel is allowed to flow into the gel before addition of the next layer.

For use in the assay, the tubing at the bottom 20 of the prepared column is connected to a peristaltic pump to control the flow rate of the assay. An appropriate dilution of test sample for iron analysis is applied to the column. The iron solution ("test" solution) is passed through the assay column at controlled flow rates, typically between 10 and 15 minutes for complete entrance. When all the solution is into the gel bed, the columns are rinsed with water. As the test solution flows through, color develops in some of the Ferene-containing reaction zones. The 30 number of colored zones resulting is a function of the concentration of iron in the test solution.

Example II

A. The enzyme cholinesterase reacts with and is inhibited by toxic organophosphate and carbamate agents. Cholinesterase and the chromogenic sulphydryl

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1 reagent 5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's
Reagent) are immobilized upon agarose beads, which are
then assembled into columns according to Example I. A
test solution (diluted blood serum) is added to the
column and migrates through the reaction zones follow-
ing which a solution ^{of butyryl-thiobutyric} ~~of butyryl-thiobutyric~~ iodide is
B added. The zones retaining catalytically active
cholinesterase will generate a yellow color through
reaction of the thiobutyrine produced by the hydrolytic
activity of the cholinesterase, with the immobilized
10 Ellman's Reagent. The presence of reactive choline-
sterase-inhibiting toxin in the test sample will re-
sult in fewer colored bands, which will be in the
downstream end region of the column.

B. Amino acids and other nucleophilic amine
compounds are measured by use of the chromogenic re-
agent 2,4-dinitrofluorobenzene (FDNB), which produces
a yellow product upon reaction. A 0.1 ml aqueous
sample, containing about 0.1-1.0 micromoles of amino
analyte, is transferred to a siliconed glass vessel.
20 The pH is adjusted to 7.0, if necessary, and two
milligrams (25 micromoles) of NaHCO₃ is added and
dissolved. Next is added 0.12 ml of 0.15% FDNB in
absolute alcohol (1.5 micromoles). This solution is
prepared fresh shortly before use. After the reaction
has neared completion, it is analyzed for remaining
FDNB (reactant) content by flow exposure to the assay
system prepared according to Example I. In this case,
a similar amine-containing analyte is immobilized in
the zones in a permeable solid medium at a known con-
tent (e.g., 0.1-0.25 micromoles per reaction zone).
30 After rinsing with 50% ethanol in aqueous solution,
the number of yellow reaction zones produced and re-
maining after rinse will be inversely related to the
amount of analyte in the test sample.

Example III

1 The IgG fraction from rabbit anti-penicilloyl-bovine gamma globulin was partially purified by precipitation with 33% saturated ammonium sulfate. The precipitate was redissolved and dialyzed against phosphate buffered saline (PBS). This IgG preparation was used for immobilizing antibodies onto beaded agarose. The agarose was suspended in dioxane, then reacted with carbonyldiimidazole. After being washed with
10 dioxane, it was suspended in water, and then in aqueous borate buffer, pH 9.0. The IgG was then added to the activated agarose and the gel suspension stirred by rocking at 4° C for 2 days. After extensive washing with PBS, the gel containing immobilized antibody was ready for use in the assay.

20 Small columns were prepared from silanized Pasteur pipettes by breaking off both ends, attaching short pieces of tubing to the bottom (constricted) ends and inserting glass wool plugs in the column bottoms. The columns were packed by sequentially inserting alternating layers of agarose-IgG separated by layers of unmodified agarose. Typically 0.4 ml of 1:1 agarose suspension was applied directly above the plugs, followed by alternating layers of 50 microliters of a 1:1 suspension of agarose-IgG (to form the reaction zones) and 0.2 ml of agarose suspension (to form spacer layers). After each addition, the walls of the columns were rinsed with PBS and the solution above the gel was allowed to flow into the gel before
30 addition of the next layer.

For use in an assay, tubing at the bottom of the prepared column was connected to a peristaltic pump to control the flow rate of the assay. An appropriate dilution of penicilloyl-glucose oxidase ("Pen-GO") (typically 0.1 microgram Pen-GO in 1 ml PBS) with or without known amounts of the analyte

1 (penicilloyl-epsilon amino caproate) (Pen-EAC), was
 applied to the column. The Pen-GO was prepared by
 reacting penicillin G with glucose oxidase in borate
 buffer, pH 9.0, for 2-3 days at 4° C. The Pen-GO
 solution was passed through the assay column at con-
 trolled flow rates, typically between 10 and 15 min-
 utes for complete entrance into the gel. When all the
 solution was into the gel bed, a detector solution was
 added to the column. The detector solution was pre-
 pared as follows: 0.20 ml of horseradish peroxidase
 (HRP) solution (2 mg/ml), 2 ml of 18% glucose solu-
 tion, 1 ml of 0.2 M phosphate buffer (pH 6.0) and
 0.100 ml of 1% o-dianisidine was diluted 1:10 in PBS
 and 1 ml or less was applied to the columns at the
 same flow rate as the previous solutions. Brown color
 developed in some of the reaction zones. Presence of
 the penicilloyl moiety in the Pen-GO solution results
 in the upper reactive zone or zones being lighter in
 color, with color being generated in zones further
 down the column.

10 This Example may be repeated for the analysis
 of serum albumin (a large protein molecule) by re-
 placement of the penicillin-glucose oxidase conjugate
 with an albumin-glucose oxidase conjugate.

Example IIIA

20 Peroxidase-labeled IgG prepared from rabbit
 antiserum against penicillin was immobilized in small
 strips of filter paper by the method reported in Ex-
 ample III. Catalase was bound to other, similar
 strips of filter paper. The first and second men-
 tioned strips were then cut into rectangular shapes to
 provide, respectively, reaction zones and spacer lay-
 ers. The small rectangular pieces of filter paper
 were then layed onto a strip of adhesive tape, alter-
 nating the reaction zones and spacer layers with edges
 of the sequential pieces of paper overlapping or at

1 least touching one another to provide a continuous capillary flow path.

Penicilloyl-glucose oxidase ("Pen-GO") in a solution of Human Serum Albumin ("HSA") was freeze-dried inside a test tube. Within another test tube, made of brown glass for protecting the contents from light, was freeze-dried a solution of o-dianisidine and glucose in phosphate-buffered saline at pH 6.0.

10 A short wick was attached to the bottom of the prepared filter paper strip described above, and a longer wick was placed in contact with the upper end of the strip. The strips themselves can be stored under refrigeration, and preferably are retained in a wet condition resulting from the preparation described above.

20 In one example of use, a test solution consisting of a measured volume of milk containing a known concentration of penicillin G is added to the test tube containing the freeze-dried Pen-GO, and the tube is shaken gently to mix the contents. The filter paper strip is then inserted into the tube with its upper, longer wick extending over the lip of the tube and then downwardly as shown in Figure 2. When the entire solution has been taken up by the strip (or, alternatively, when the solution reaches an arbitrary flow line marked on the upper wick and designated "F" in Figure 2) the filter paper strip is removed from the test tube and is placed in the brown glass tube to which previously has been added water to dissolve the 30 freeze-dried contents thereof. The latter solution similarly wicks upwardly through the filter paper strip, causing color development to occur in certain of the reaction zones as determined by the quantity of penicillin G in the initial test solution.

In this example, the penicillin G in the milk and the penicillin of the Pen-GO compete for binding

1 sites on the antibodies immobilized in the reaction
2 zones of the filter paper strip. Of course, larger
3 concentrations of penicillin G in the milk sample
4 cause the penicillin G and the Pen-GO to migrate fur-
5 ther through the filter paper strip. The presence of
6 Pen-GO in any of the zones is indicated through the
7 development of color from the reaction of H₂O₂
8 with o-dianisidine, the H₂O₂ being formed from the
9 glucose oxidase in the presence of glucose and oxygen,
10 and as catalyzed by the peroxidase. The catalase in
11 the spacer layers catalyzes the conversion of H₂O₂
12 into O₂ and H₂O, and thus prevents migration of
13 H₂O₂ from one reactive zone to another.

14 As with each of the apparatuses described here-
15 in, the device of this example may be calibrated by
16 determining how many of the reaction zones become
17 colored as a result of the test procedure. For exam-
18 ple, one of the reaction zones may change color only
19 when the test solution (e.g., milk) contains at least
20 9 nanograms of analyte (e.g., penicillin G) per ml.
For a sample of milk containing an unknown concentra-
tion of penicillin G, one merely counts the number of
reaction zones that have changed color to find the
narrow, defined concentration range within which lies
the penicillin G concentration.

Example IV

21 Antibody against a polyvalent antigen (e.g.,
22 serum albumin) analyte is labeled with peroxidase and
23 bound to permeable solid medium according to Example
III to form reaction zones in a column. Another batch
of the same or similar antibody is labeled with an
enzyme such as glucose oxidase. Into the column is
poured a test sample containing an unknown amount of
analyte antigen. Through the column is then flowed
the soluble glucose oxidase-antibody in the presence

1 of glucose plus catalase plus o-dianisidine. The num-
ber of colored bands resulting is directly related to
the amount of analyte antigen in the test sample rela-
tive to the antigen binding capacity of the antibody
zones. In this example, the antigen first reacts with
the bound antibody and binds to the antibody, forming
a predetermined product. The latter, in turn, is de-
tected by the coupling of the glucose oxidase antibody
conjugate to available antigenic sites on the antigen
followed by the color forming reaction.

10 Example V

An analyte or a derivative thereof (e.g., peni-
cillin-peroxidase) is covalently bound to a permeable
solid medium according to Example III. An enzyme-la-
beled receptor (e.g., glucose oxidase-antibody against
penicillin) is prepared and exposed to the immobilized
analyte to form the specific binding complex (e.g.,
immune complex). The assay unit is assembled accord-
ing to Example III. Subsequent exposure to a test
sample containing an unknown amount of analyte is done
at elevated temperature (e.g., 60° C) to hasten the
attainment of equilibrium through competitive binding
of the immobilized analyte and analyte in the test
sample with the enzyme-labeled antibody. Analyte in
the test sample under such conditions will competi-
tively displace the labeled antibody from the immobi-
lized analyte. The number of colored reaction zones
resulting from the procedure is inversely related to
the amount of analyte in the test sample. These bands
will appear in the terminal or downstream portion of
the column.

20 Example VI

Three assay columns with 4 reaction zones each
were prepared according to Example III, except that
the top reaction zone was prepared with 75 microliters
of IgG-agarose suspension (1:1) and the lower 3 zones

1 with 50 microliters. Test samples containing 0, 50
and 200 nanograms Pen-EAC, were placed in different
columns, with each test sample containing 200 ng Pen-GO
per ml. Flow time for sample application was 20 min.
Application of the solution of signal generating re-
agents produced 2 colored zones with the 0 ng Pen-EAC
sample, 3 in the 50 and 4 with the 200 ng sample.

10 A wider and more precise range of analyte con-
tent, of course, may be measured by using a larger
number of assay zones.

20 In a preferred embodiment, only a single pass
through the apparatus of a single liquid material is
required. An analyte may be mixed with an analyte
derivative, chromogen or other material and flowed
through the apparatus to yield an appropriate test
result. In a further preferred embodiment, the ap-
paratus is chemically complete in that it includes all
reactants and other chemicals necessary or desirable
for the quantitative analysis of an analyte; that is,
all that is required is that the analyte in a liquid
carrier be flowed through the apparatus. Elements of
the apparatus that, if combined, would undergo reac-
tion in the absence of the analyte may be maintained
in different zones. For example, the bottom-most
layer (20.2) of the strip of Figure 2 may contain a
reactant physically separated from reactants in the
adjacent reaction zone. When the analyte in a carrier
liquid is flowed through the layer (20.2), the reac-
tant in this layer together with the analyte and car-
rier liquid is flowed into the first reaction zone.
If desired, a reactant may be provided in the form of
a solid and may merely be placed upon the upper layer
(18.4) of the column of Figure 1, the reactant being
dissolved by and carried with the liquid carrier and
analyte into the column.

1 The above-described embodiments are typified by
the following Examples VII-IX which also describe and
exemplify a preferred format of the invention.

10 This format requires at least two enzymes, one
of which is coupled to an analyte to form an analyte
derivative and catalyzes a color-forming reaction, and
another enzyme that is immobilized in reaction zones
which also contain antibody to the analyte, the latter
enzyme providing substrate for the color-generating
enzyme. In this format, therefore, only a single
solution which consists of or contains the analyte
test sample is flowed into or through the solid medium
after which color develops in the reaction zones, the
number of colored zones being directly related to the
concentration of analyte in the test sample.

20 Example VII

20 The IgG fraction from rabbit anti-penicilloyl-
bovine serum albumin was partially purified by precip-
itation with 33% saturated ammonium sulfate. This pro-
tein was coupled to microcrystalline cellulose by re-
action of the cellulose with carbonyldiimidazole in
dioxane, followed by washing and then by reaction with
the IgG preparation in borate buffer at pH 9.0 at 4
degrees C for two days. The cellulose was then
washed extensively with PBS and used for preparation
of banded strips. Glucose oxidase was also coupled to
microcrystalline cellulose in the same manner. A
penicilloyl-peroxidase was prepared by first coupling
a polyacrylamide amine to HRP, then reacting penicil-
lin G with that preparation. It is believed that the
use of a linear polymer as a spacer for attaching the
hapten to the enzyme allows more hapten molecules to
be coupled to each enzyme molecule and renders the
hapten molecules more accessible for binding to anti-
body, thus speeding the binding rate. Polyacrylamide
was synthesized by dissolving 0.5 gm. of acrylamide in

1 200 ml. of deionized water, degassing, then adding 0.2
ml. of N,N,N',N'-tetramethylethylenediamine and 0.15
gm. of ammonium persulfate. This solution was mixed,
then allowed to sit at room temperature for 30 min.
then passed through an ultrafiltration membrane,
dialyzed vs. deionized water and lyophilized. The
polyacrylamide was then dissolved in 1.0 ml. of 0.2 M
phosphate buffer at pH 7.7 and 0.3 ml. of 25% glutar-
aldehyde was added. This solution was incubated at 37
10 degrees C for 19 hours after which it was passed
through a Sephadex^{*}G-25 column to remove the excess
B glutaraldehyde. The void volume fractions which ab-
sorbed strongly at 230 nm were pooled and added to a
solution of diaminodipropylamine (0.5 ml. in 2.0 ml.
of water) at pH 9.0. This solution was allowed to
react at 4 degrees C over night. The reaction mixture
was then passed through a Sephadex^{*}G-150 column and
the fractions that absorbed significantly at 230 nm.
were divided into four pools of equal volume, the
20 second of which was coupled to peroxidase (HRP). HRP
was reacted with 1.25% glutaraldehyde at pH 7.0
for 15 hours at room temperature. After passing the
reaction mixture through a Sephadex^{*}G-25 column, the
HRP-containing fractions were pooled and added to the
polyacrylamide-diamine preparation, the pH was ad-
justed to 9.0, and this solution was allowed to react
at 4 degrees C overnight. The peroxidase-polyacryla-
mide-diamine was then passed through a Biogel^{*}P-100
column and the void volume fractions were pooled and
concentrated, then reacted with penicillin. Fifty mg.
30 of penicillin G was added to the peroxidase-polyacry-
lamide-diamine, the pH adjusted to 9.0 and stirred at
4 degrees C over night. This preparation was then
dialyzed extensively, then used for the assay.

* TRADE MARK

1 Banded strips were prepared by cutting 0.5 X
8.0 cm. strips of a polyester film having a hydrophilic
B surface onto which were glued strips of Whatman^{*3MM}
chromatography paper. At one end was glued a 0.5 X 4.0
cm. long paper strip followed by a 3.5 mm. space. Then
three one cm. long paper strips were glued onto the
A ^{Polyester} ^{Film} Mylar strip with 2.0 mm. spaces between them. The
paper on the ^{Polyester Film} Mylar was wetted with a solution of 0.02%
o-dianisidine in water. The spaces were then filled in
10 with a suspension of microcrystalline cellulose pre-
pared by mixing 50% suspensions of the IgG-cellulose
and the glucose oxidase-cellulose in a 20:1 ratio.
The first space was filled with 20 ul. of this suspen-
sion and the other three spaces each contained 10 ul.
These strips were air dried, then stored dry until
used.

20 The strips were developed by placing the end
with the longer paper spacer into a small vial con-
taining the developing solution. This solution
contained peroxidase-polyacrylamide-diamine-penicillin
(25 ul. of a 0.25 microgram/ml. solution), glucose
(0.3 ml of a 1.125% glucose solution in 0.2M phosphate
buffer at pH 6.0) and 10 ul. of dilutions of penicil-
loyl-aminocaproic acid (EAC) in water. Under these
conditions, pink bands could readily be observed after
20-30 min., such that, with no penicilloyl-EAC in the
developing solution, one band was colored; with 0.4
micromolar hapten (penicilloyl-EAC), two bands were
colored; and with 1.0 uM penicilloyl-EAC, all three
bands were colored.

30 If needed or desired, antibody to peroxidase,
an HRP-binding lectin or some other binder or inacti-
vator of peroxidase can be included in the spacer lay-
ers for the purpose of improving the sharpness or
decisiveness of zone color determinations. Further-
more, catalase immobilized in the spacer layers may

*TRADE MARK

1 permit more rapid color development in the reaction
 zones without generation of color in the spacer layers.

Example VIII

Banded strips are prepared according to Example VII, except that all of the components of the assay except the sample to be tested are incorporated into the strip. The peroxidase-polyacrylamide-diamine-penicillin is dissolved in a solution of between 0.5 and 1.0% gelatin containing 2.5% glucose and 0.2 M phosphate buffer at pH 6.0, 0.1 ml. of which is applied to the bottom paper strip and dried. In this example, therefore, the user has only to dip the strip into a solution suspected of containing the analyte, wait for a prescribed time, then read the results by counting the number of colored bands on the strip.

Example IX

Assay columns are prepared according to Example III, except that the reaction zones are composed of a mixture of IgG-agarose and glucose oxidase-agarose (20:1). Peroxidase-penicillin (as prepared in Example VII), glucose, o-dianisidine, and phosphate buffer, stored in dry form, are dissolved in 1.0 ml of the test sample which is then added to the column and allowed to flow through. The results are read after the prescribed time by counting the number of colored bands on the column. The reagents added to the analyte test sample can be in the form of a small pellet or can be dried onto the under surface of the cap for a small vessel used to measure the volume of sample, etc. In the latter case, the vessel is filled, the cap placed on top, the vessel inverted a few times and the sample is poured into the column. The reagents to be mixed with the sample can even be dried onto a small plug that is stored in the top of the column, in which case they dissolve when the sample is added to the column.

1 Various other enzyme pairs can be used for generating color in the reaction zones. For example, alkaline phosphatase can be immobilized in the reaction zones with beta-galactosidase coupled to the analyte. The use of naphthol-beta-D-galactopyranoside-6-phosphate as substrate for the alkaline phosphatase results in the generation of naphthol-beta-D-galactopyranoside, which is hydrolyzed by beta-galactosidase to produce naphthol which in the presence of a diazonium salt results in a colored product in the reaction zones.

10
20
30 The accuracy and reliability of the apparatus of the invention depends to some extent upon how readily or easily the generation of color or other detectable change in the different reaction zones may be ascertained. A reaction zone in the direction of analyte flow desirably should show detectable changes only when a significant, minimum quantity of analyte or other material being detected has passed through the preceding reaction zone; since the physical nature of the apparatus often does not permit reaction to go fully to completion in each such zone, a small "tail" e.g., trace, amount of material may flow into successive zones and may be marginally detected in such zones to yield readings that are difficult to interpret. One may largely avoid this problem, however by several means. Detectors may be employed that are sensitive only to minimum concentrations of a chemical moiety to be detected. For example, one may utilize o-phenylene diamine in place of o-dianisidine as a chromophore in the above examples, the former being less sensitive. Another method involves the placement in spacer layers or, less desirably, in reaction zones, of small quantities of "scavenger" reactants capable of immobilizing or deactivating

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1 trace amounts of materials, as exemplified in Example
VII. This enables the sensitivity and operation of
the apparatus to be tailored as desired to particular
analyses. Control of sensitivity and reliability also
may depend upon the concentration of the reactant in
the solid reaction zones, and the solubility of mate-
rials such as the colored product in some analyses.

10 While a preferred embodiment of the present in-
vention has been described, it should be understood
that various changes, adaptations and modifications
may be made therein without departing from the spirit
of the invention and the scope of the appended claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR
PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. Apparatus for the quantitative analysis of an analyte in a fluid, comprising a fluid-permeable solid medium defining a path for fluid flow and having a predetermined number of successive, spaced reaction zones in the path of flow, the reaction zones having immobilized therein predetermined quantities of a reactant capable of competitively reacting with or competitively binding to the analyte or an analyte derivative to result in the formation of a predetermined product; the analyte or its derivative, the reactant or the predetermined product in the reaction zones being detectable and the number of reaction zones in which such detection occurs indicating quantitatively the amount of analyte in said fluid.
2. The apparatus of claim 1 wherein said fluid-permeable solid medium includes spacer sections in flow communication with but separating said reaction zones.
3. The apparatus of claim 1 wherein the fluid-permeable medium in the reaction zones has immobilized therein an antibody specific to an analyte to be quantitatively analyzed.
4. The apparatus of claim 1 including detection means for detecting, in the reaction zones, the analyte or its derivative, the reagent, or the predetermined product.
5. The apparatus of claim 2 wherein said reaction zones are vertically spaced in a column.
6. The apparatus of claim 2 wherein the reaction zones are spaced along the length of a strip of solid, fibrous material through which fluid is drawn by capillary action.
7. The apparatus of claim 2 wherein the reaction zones are in the shape of concentric rings and the spacer sections are in the shape of concentric rings sandwiching between them the reaction zones.
8. The apparatus of claim 2 including flow rate controlling means for controlling the rate of fluid flow along the fluid flow path.
9. Method for the quantitative analysis of an analyte in a carrier liquid, comprising the steps of:
providing a liquid-permeable solid medium defining a flow path and having a predetermined number of successive, spaced reaction zones in

the path of flow, said reaction zones having immobilized therein a reactant competitively reactive with or competitively binding to the analyte or an analyte derivative or both to result in the formation of a predetermined product;

flowing said liquid along the flow path and sequentially through the spaced reaction zones; and

detecting the presence of analyte, analyte derivative, reactant or predetermined product in the reaction zones;

the amount of analyte in the determining liquid being a function of a number of zones in which such detection occurs.

10. The method of claim 9 wherein said analyte and said reactant are ones of a specific ligand-antiligand binding pair.

11. The method of claim 10 wherein said reactant includes an antibody specific to said reactant.

12. The method of claim 9 wherein reaction between said analyte and reactant renders the analyte, analyte derivative, reactant or predetermined reaction product nondetectable during said detection step.

13. The method of claim 9 wherein said liquid contains said analyte and a known concentration of said analyte labeled with a chemical moiety, the presence of which chemical moiety is detected in said detection step.

14. The method of claim 13 wherein the chemical moiety is an enzyme.

15. The method of claim 13 wherein the liquid is milk, the analyte is penicillin and the reactant includes an anti-penicillin antibody.

16. The method of claim 15 wherein the liquid includes a known concentration of an enzyme-labeled penicillin.

17. Method for the quantitative determination of an analyte in a fluid containing a known quantity of a labeled analyte, comprising the steps of:

providing a fluid-permeable solid medium defining a fluid flow path and having immobilized therein a reactant reactive with the labeled analyte in competition with the analyte to result in the formation of a predetermined product;

flowing said fluid along the flow path; and

detecting the presence of the labeled analyte or the predetermined product along the flow path;

the length of the flow path in which such detection occurs being a

function of the amount of analyte in the fluid.

18. Method for the quantitative determination of penicillin in a liquid containing a known quantity of a labeled penicillin, comprising the steps of: providing a liquid-permeable solid medium defining a liquid flow path and having immobilized therein a reactant including an antibody to said penicillin and to said labeled penicillin and reactive competitively therewith to result in the formation of a predetermined product; flowing said liquid along said flow path; and detecting the presence of the labeled penicillin or the predetermined product along the flow path; the length of the flow path in which such detection occurs being a function of the amount of penicillin in the liquid.

19. The apparatus of claim 1 including means in the reaction zones or spacer sections for suppressing therein the detection of trace amounts of the analyte or its derivative, the reactant or the predetermined product.

20. The apparatus of claim 3 wherein said reaction zones are particularly adapted to change colour upon contact therein with an analyte derivative that is a conjugate of an analyte and an enzyme, the reaction zones having immobilized therein another enzyme, one of said enzymes being capable of generating substrate for the other said enzyme.

21. The apparatus of claim 20 wherein said analyte-enzyme conjugate is contained in a layer upstream from a reaction zone, whereby flow of an analyte in a fluid carrier through said layer carries the analyte conjugate into said reaction zone.

22. The method of claim 9 wherein only a single liquid containing an analyte is flowed in said flow path.

23. The method of claim 22 wherein said liquid consists essentially of the analyte and carrier liquid.

24. The method of claim 22 wherein said liquid includes an analyte and an analyte derivative.

25. The method of claim 9 including the step of suppressing the detection of trace amounts of analyte, analyte derivative, reactant or predetermined product.

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